Characterization of *hgpA*, a gene encoding a haemoglobin/haemoglobin–haptoglobin-binding protein of *Haemophilus influenzae*

Hongfan Jin,^{1,2} Zhen Ren,^{1,2} Paul W. Whitby,¹ Daniel J. Morton¹ and Terrence L. Stull^{1,2}

Author for correspondence: Terrence L. Stull. Tel: +1 405 271 4401. Fax: +1 405 271 8710.
e-mail: Terrence-Stull@ouhsc.edu

Departments of Pediatrics,
CHO 2308, 940 Northeast
13th Street¹ and
Microbiology/Immunology²,
University of Oklahoma
Health Sciences Center,
Oklahoma City, OK 73104,
USA

***Haemophilus influenzae* binds haemoglobin and the haemoglobin–haptoglobin complex and utilizes either as a sole source of haem. Previously, a DNA fragment was cloned from *H. influenzae* that encodes an approximately 120 kDa protein (HgpA) expressing haemoglobin-binding activity in *Escherichia coli*. Partial sequence analysis revealed significant homology of HgpA with other bacterial haem- and iron-utilization proteins, and a length of CCAA repeating units immediately following the nucleotide sequence encoding the putative leader peptide. In the present study, the complete nucleotide sequence of the cloned DNA fragment was determined and the sequence was analysed. In addition to homology with other haem- and iron-utilization proteins, seven regions typical of TonB-dependent proteins were identified. The transcript of *hgpA* was determined to be monocistronic by RT-PCR. PCR performed with different colonies of a single *H. influenzae* strain at one CCAA-repeat-containing locus indicated varying lengths of CCAA repeats, suggesting that haemoglobin and haemoglobin–haptoglobin binding in *H. influenzae* is regulated by strand slippage across CCAA repeats, as well as by haem repression. *E. coli* containing cloned *hgpA* bound both haemoglobin and the haemoglobin–haptoglobin complex. A deletion/insertion mutation of *hgpA* was constructed in *H. influenzae* strain HI689. Mutation of *hgpA* did not affect the ability of *H. influenzae* either to bind or to utilize haemoglobin or haemoglobin–haptoglobin following growth in haem-deplete media. Affinity purification of haemoglobin-binding proteins from the mutant strain revealed loss of the 120 kDa protein and an increased amount of a 115 kDa protein, suggesting that at least one additional haemoglobin-binding protein exists.**

Keywords: *Haemophilus influenzae*, haemoglobin, haemoglobin-binding protein, haem acquisition, outer-membrane protein

INTRODUCTION

The human-specific pathogen *Haemophilus influenzae* causes a range of infections including otitis media, meningitis, epiglottitis and pneumonia (Turk, 1984). The incidence of invasive disease caused by *H. influenzae* strains with the type b capsule has been radically reduced by vaccines based on the type b capsular polysaccharide (Madorne *et al.*, 1990; Murphy *et al.*, 1993). Currently

available vaccines based on the type b capsule are not protective against disease caused by unencapsulated strains of *H. influenzae*, and such strains are a significant cause of otitis media in childhood, neonatal sepsis and pneumonia in adults (Falla *et al.*, 1993; Wallace *et al.*, 1978; Klein, 1997).

H. influenzae has an absolute growth requirement for protoporphyrin IX, the immediate precursor of haem (Evans *et al.*, 1974). *In vitro*, the requirement may be satisfied by haem, haemoglobin, and complexes of haemoglobin–haptoglobin, haem–haemopexin and haem–albumin (Stull, 1987). *In vivo*, haem is intra-

The GenBank accession number for the sequence determined in this work is U51922

cellular, in the form of haemoglobin or haem-containing enzymes and thus unavailable to invading micro-organisms (Bezkorovainy, 1987; Lee, 1995). Haemoglobin released by erythrocytes is avidly bound by the serum protein haptoglobin, and the haemoglobin-haptoglobin complex is rapidly cleared by hepatocytes (Bezkorovainy, 1987; Otto *et al.*, 1992). Free haem, principally derived from the degradation of methaemoglobin, is bound by the serum proteins haemopexin and albumin and is also cleared from the circulatory system by hepatocytes (Bezkorovainy, 1987). We have shown that *H. influenzae* binds haemoglobin, possibly as an initial step in the utilization of haemoglobin-associated haem (Frangipane *et al.*, 1994), and we have cloned a DNA fragment encoding an approximately 120 kDa haemoglobin-binding protein (HgpA) of *H. influenzae* (Jin *et al.*, 1996).

The objective of the current study was to characterize the gene encoding the previously identified 120 kDa haemoglobin-binding protein (HgpA) of *H. influenzae* (Jin *et al.*, 1996).

METHODS

Bacterial strains, culture conditions and plasmids. *H. influenzae* type b strain HI689 has been previously described (Jin *et al.*, 1996). *H. influenzae* Rd KW20 (ATCC 51907), used in the complete sequencing of the *H. influenzae* genome (Fleischmann *et al.*, 1995), was obtained from the American Type Culture Collection (ATCC). An additional Rd strain maintained in this laboratory was used in certain experiments. Non-typable *H. influenzae* TN106 has been described by Maciver *et al.* (1996) and was kindly provided by Eric Hansen, University of Texas Southwestern Medical Center, Dallas, TX, USA. *H. influenzae* type b strain HI1715 is a strain shown by Southern analysis to have a single CCAA-repeat-containing locus associated with a gene which is highly homologous to *hgpA* (data not shown). *H. influenzae* was routinely maintained on brain heart infusion (BHI) agar (Difco) supplemented with 10 µg haem ml⁻¹ and 10 µg β-NAD ml⁻¹. Haem-restricted growth of *H. influenzae* was performed in BHI broth supplemented with 10 µg β-NAD ml⁻¹ and 0.1 µg haem ml⁻¹. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) medium. Ribostamycin was added where appropriate to a final concentration of 15 µg ml⁻¹ for *H. influenzae* and 50 µg ml⁻¹ for *E. coli*. Plasmids pHFJ2 and pUC19::TSTE, containing the aminoglycoside resistance cassette TSTE, have been previously described (Jin *et al.*, 1996; Sharetsky *et al.*, 1991). Clones generated in the sequencing of the Rd KW20 genome (Fleischmann *et al.*, 1995) encompassing the ORFs designated HI0588–HI0592 were obtained from the ATCC.

DNA methodology. Restriction endonucleases were obtained from New England Biolabs and were used as directed by the manufacturer. Genomic DNA was isolated using the DNA Now reagent (Biogentex) as directed by the manufacturer. Plasmid DNA was isolated using Qiagen plasmid kits according to the manufacturer's recommendations. Sequencing of double-stranded template DNA was performed by automated sequencing on an ABI model 373A sequencer at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater, OK, USA. Oligonucleotides were synthesized at the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA.

Southern analysis was performed as described by Sambrook *et al.* (1989). The Enhanced Chemi-Luminescence (ECL) random prime labelling kit (Amersham) was used as directed by the manufacturer to label DNA fragments. Hybridization was performed overnight at 60 °C and stringency washes were 1 × SSC, 0.1% (w/v) SDS for 15 min and 0.5 × SSC, 0.1% SDS for 15 min, both at 60 °C (1 × SSC: 8.8 g NaCl l⁻¹, 4.4 g sodium citrate l⁻¹, pH 7.0). Hybridized probes were detected using ECL nucleic acid detection reagents (Amersham) as directed by the manufacturer.

Construction of an insertion/deletion mutation of *hgpA*. Mutation of *hgpA* was achieved as follows. Plasmid pHFJ2 (Jin *et al.*, 1996) was completely digested with *Bgl*II and separated on a 1% (w/v) agarose gel. The 2.5 kbp internal *Bgl*II fragment was deleted and the remaining 4.5 kbp fragment was purified using the Prep-a-Gene kit (Bio-Rad). The 2.2 kbp TSTE fragment from pUC19::TSTE (Sharetsky *et al.*, 1991) was excised using *Bam*HI and gel-purified. TSTE was ligated to the 4.5 kbp *Bgl*II fragment of pHFJ2, yielding plasmid pHFJC (Fig. 1). The plasmid pHFJC was transformed into *H. influenzae* strain HI689 made competent using the MII medium of Spenser & Herriott (1965). Recombinant colonies were initially selected by growth on BHI agar containing ribostamycin (15 µg ml⁻¹). The mutant strain was designated HI689*hgpAΔBgl*II. Confirmation of the appropriate chromosomal rearrangements was obtained by Southern blot analysis.

Cloning of DNA downstream of *hgpA*. The sequence of the insert of pHFJ2 was analysed, and a putative gene (*hgpA*) was identified based on the existence of an ORF and homology to other sequences in the databases. Since a cloned *Eco*RI fragment was used to generate pHFJ2 (Jin *et al.*, 1996), the ORF ended with GAATTC, encoding glutamic acid (E) and phenylalanine (F). Sequence comparison with other homologous haem- and iron-utilization proteins suggested a stop codon following the nucleotides encoding EF. To confirm the presence of a stop codon, the region downstream of the proposed ORF was cloned. The primers HFJ25 and HFJCTERMS (Table 1) were used in the PCR to amplify a 315 bp DNA fragment encoding the C terminus of *hgpA*. This gene fragment was used as a probe. HI689 chromosomal DNA was digested with *Eco*RV, and Southern blot analysis was performed using the 315 bp DNA probe. The probe hybridized with a DNA fragment of approximately 1.4 kbp. Restriction mapping of *hgpA* indicated an *Eco*RV site internal to *hgpA* (Fig. 1), approximately 0.6 kbp upstream of the C terminus; thus the 1.4 kbp hybridizing fragment would contain about 0.8 kbp downstream of the C terminus of *hgpA*. An HI689 mini-library was constructed by gel-purification of the DNA fragment of approximately 1.4 kbp in an *Eco*RV digest of HI689 chromosomal DNA and ligation of the purified fragments to the pCR-Blunt vector (Invitrogen). The library was screened with the 315 bp probe and a positive clone was identified and designated pHFJC'.

PCR amplification across a region of CCAA repeats. A pair of oligonucleotide primers designated PBUS and PBDS were designed 5 bp upstream and downstream of the CCAA repeats of HI1715, respectively (Table 1). Following repeated subculture, single colonies of *H. influenzae* HI1715 were picked from chocolate agar plates and chromosomal DNA extracted using the Instagene kit (Bio-Rad) as directed by the manufacturer. Using HI1715 chromosomal DNA as template, the PCR amplification was performed in a total volume of 50 µl containing 5 µl of 10 × 30 mM Mg PCR buffer (Idaho Technology), [α-³²P]dATP (10 µCi; 3.7 × 10⁵ Bq), 1 µl 10 mM dNTPs, 1 U *Taq* DNA polymerase and 10 pM of each primer. PCR was performed in a RapidCycler thermocycler (Idaho

Fig. 1. Partial restriction map of the insert of pHFJ2, the deletion/insertion derivative pHFJC and the clone encompassing the C-terminal region of *hgpA* and downstream sequence, pHFJC'. The coding sequence of *hgpA* is shown by the boxed area. The TST insertion element encoding ribostamycin resistance is shown by the black area. The scale indicates size in kbp.

Table 1. Primers used in this work

Name	Sequence (5'–3')	Primer location
PBUS	GGGCTAACGGCAAGTGTGCTT	5 bp upstream of cca repeats
PBDS	GTTCTAGTTGTTGTTTCAGAAGCATCC	5 bp downstream of cca repeats
HFJ2F4	GAAGTACGCATAGAAATGATGC	Intergenic region between HI0594 and <i>hgpA</i>
HFJ16	CGG GCAATTTTGGCGGAGC	<i>hgpA</i>
HFJ25	TATATCTCTCATGCATCAG	<i>hgpA</i>
HFJCTERMS	GAATTCAACTGAAGTAC	<i>hgpA</i>
HFJ26	GTCAGTTTCAAGTTGAATTC	<i>hgpA</i>
HFJ29	CATATTCATCAAAAGTGCGG	HI0587
HFJXN	GACCAGGGATCCAATAGTAATGCTTCT	<i>hgpA</i>
HFJXC	GGAAGGGGTACCTTAGAATTCAAAGT	<i>hgpA</i>

Technology). The reactions were cycled 30 times with an annealing step of 58 °C for 10 s, extension at 72 °C for 30 s and a denaturing step at 95 °C for 10 s. PCR products were visualized by running on a 6% denaturing polyacrylamide urea gel, dried and exposed to X-ray film as required. Sizing of the PCR products was achieved by running a sequencing reaction in lanes adjacent to the PCR products.

RT-PCR. This was performed essentially as described previously (Whitby *et al.*, 1997). RNA (1 µg) prepared from haem-starved HI689 grown to mid-exponential phase (OD_{605} 0.64) using the RNeasy total RNA kit (Qiagen) as directed by the manufacturer was treated with 1 U DNase I (Gibco-BRL) in a total volume of 12.5 µl for 15 min at room temperature. The DNase I was inactivated by the addition of 1 µl 25 mM EDTA solution to the reaction mixture and heating for 10 min at 65 °C. A 12.5 µl aliquot of the DNase-treated RNA sample was used for annealing with 1 µl random nonamer primer (Amersham random prime labelling kit) by heating to 70 °C for 2 min, followed by rapid chilling on ice. cDNA was synthesized at 42 °C using Superscript II reverse transcriptase (Gibco-BRL) in a 20 µl reaction containing 100 U enzyme, 1 ×

first-strand buffer, 2.5 mM of each dNTP (dATP, dCTP, dGTP, dTTP) and 5 U RNase inhibitor. Reverse transcriptase was omitted in negative-control tubes. After 1 h, the reaction mixture was heated at 94 °C for 5 min and subsequently stored at –20 °C. The cDNA or the negative-control reaction (3 µl) was used as template in the PCR. In addition, reactions using either HI689 chromosomal DNA or ddH₂O as template were used as positive and negative controls, respectively. Three sets of primers were designed for PCR (Table 1). Primer pair 1 comprised HFJ2F4 and HFJ16, primer pair 2 comprised HFJ25 and HFJCTERMS, and primer pair 3 comprised HFJ26 and HFJ29. PCR was performed for 30 cycles with the following parameters: for primer pair 1, each cycle consisted of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 45 s; for primer pair 2, each cycle consisted of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 20 s; for primer pair 3, each cycle consisted of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 45 s. The reactions were analysed on a 2% agarose gel.

Growth studies. Plate bioassays of haem source utilization

were performed on haem-deplete *H. influenzae* strains as previously described (Stull, 1987).

Dot-blot assay for haemoglobin-haptoglobin binding. A haemoglobin-haptoglobin dot-blot assay was performed as previously described (Jin *et al.*, 1996). Haptoglobin was complexed with biotinylated haemoglobin as previously described (Stull, 1987). The complex equivalent to 500 ng haemoglobin ml⁻¹ was used in the dot-blot assay.

Affinity isolation of haemoglobin-binding proteins. Outer-membrane proteins were subjected to haemoglobin affinity purification as previously described (Jin *et al.*, 1996). Eluted proteins were separated by SDS-PAGE on 7.5% acrylamide gels using the discontinuous buffer system of Laemmli (1970). Approximately 30 µl of protein preparation, representing proteins isolated from approximately 10⁶ organisms, was loaded per lane.

RESULTS

Sequencing of *hgpA* and analysis of the deduced HgpA amino acid sequence

The insert of the recombinant plasmid, pHFJ2, encoding the previously identified haemoglobin-binding activity of *H. influenzae* (Jin *et al.*, 1996), was 4358 bp as determined by automated nucleotide sequencing. Analysis of pHFJ2 and the downstream region cloned in pHFJC' indicated an ORF of 3234 bp encoding a putative protein of 1077 amino acids with a molecular mass, after cleavage of the proposed leader sequence, of 120296 Da, which is consistent with the size of the affinity-purified protein. The predicted haemoglobin-binding protein (HgpA) encoded by the gene *hgpA* showed significant homology with other bacterial iron- and haem-related outer-membrane proteins, particularly across the seven regions considered typical of

TonB-dependent outer-membrane proteins (Lundrigan & Kadner, 1986).

The genomic sequence of *H. influenzae* strain Rd KW20 reported by Fleischmann *et al.* (1995) contains four putative genes designated HI0635, HI0661, HI0712 and HI1566, which have significant homology with *hgpA*. The accession numbers of the GenBank entries corresponding to these four genes are U32746, U32749, U32754 and U32831, respectively. One particularly interesting feature of *hgpA* and its Rd KW20 homologues is a length of CCAA repeating units directly following the nucleotide sequence encoding the putative leader sequence (Table 2). We proposed that strand slippage across the CCAA region may be a mechanism of regulation (Jin *et al.*, 1996), similar to that of the *H. influenzae* *lic* locus, where slippage across CAAT repeats places potential initiation codons in or out of frame with the ORF of *H. influenzae* lipo-oligosaccharide (Weiser *et al.*, 1989; Szabo *et al.*, 1992). In the *hgpA* clone a full-length protein is encoded by the ORF; however, addition or loss of CCAA repeat units could change the reading frame and result in introduction of stop codons downstream of the CCAA repeat region (Fig. 2). To determine if variation in length over the CCAA region occurs in *H. influenzae*, PCR reactions were performed on chromosomal DNA across this region. Strain HI689 from which *hgpA* was cloned contains three genes which have lengths of CCAA repeating nucleotides immediately following the leader-peptide-encoding sequence. All three of these genes are highly homologous to each other and it has proven impossible to design primers which will amplify a *hgpA*-specific product of an appropriate size to demonstrate a change in CCAA length which may be as small as four nucleotides. Because of these complications, we selected strain HI1715, which con-

Table 2. Comparison of HgpA with the proteins encoded by ORFs HI0635, HI0661, HI0712 and HI1566 in strain Rd and with HhuA

Gene/locus	Strain cloned from:	Binding activity*	% Homology to HgpA from strain HI689 (identity/similarity)†	Predicted prototypic gene product size (kDa)‡	No. of ccaa repeats	In/out of frame as cloned
<i>hgpA</i>	HI689	Hb/Hb-Hp	100/100	120	33	In
HI0635	Rd KW20	ND	49/65	119	21	Out
HI0661	Rd KW20	ND	59/74	112	20	Out
HI0712	Rd KW20	ND	49/67	121	37	In
HI1566	Rd KW20	ND	54/72	112	19	Out
<i>hhuA</i>	TN 106	Hb-Hp	84/90	117	13	Out§

ND, Not determined.

* Hb, haemoglobin; Hb-Hp, haemoglobin-haptoglobin complex.

† Percentage homology to HgpA is given as identical amino acids and functionally similar amino acids, based on translation of the sequence subsequent to the final ccaa repeat unit. Percentage homologies were generated using the GAP program in the GCG Program Package (Devereux *et al.*, 1984).

‡ Gene product size was calculated following alteration of the number of ccaa units as appropriate and cleavage of the putative leader sequence.

§ Out of frame with the alternative start codon proposed in this paper.

Fig. 5. (a) Comparison of the organization of *H. influenzae* strain HI689 at the *hgpA* locus (top) with the organization of the Rd KW20 genome (bottom) based on nucleotide sequence analysis, showing that *hgpA* is not present at this locus in the Rd KW20 genome. HI0592 and HI0594 are ORFs from the Rd KW20 genome with no assigned function (Fleischmann *et al.*, 1995). HI0593 is upstream of HI0594. (b) Southern blot probed with the labelled clone GHIFY44. Lanes: 1, labelled λ HindIII digest; 2, Rd KW20 genomic DNA digested with *Eco*RI; 3, HI689 genomic DNA digested with *Eco*RI; 4, TN106 genomic DNA digested with *Eco*RI; 5, *H. influenzae* Rd genomic DNA digested with *Eco*RI.

and HI0592 in RdKW20 (Fleischmann *et al.*, 1995). Regions homologous to the 49 bp direct repeat upstream of *hgpA* can be identified upstream of ORFs HI0635, HI0661 and HI1566 (data not shown). The function of this direct repeat is unknown, although it may be an element regulating expression of these genes. Additional regulatory elements may include a putative fur box identified 16 bp upstream of the ATG start codon of *hgpA*. The fur box is a conserved DNA sequence which binds the ferric uptake regulator (Fur) protein and is implicated in regulation of expression of iron- and/or haem-uptake-related proteins (Bagg & Neilands, 1987), although a specific role for Fur in the regulation of *hgpA* has not been demonstrated.

Analysis of the *hgpA* transcript

The sequences flanking *hgpA* indicated the presence of a putative promoter region. The hairpin structure and a poly(T) tract following the stop codon indicated a rho-

Fig. 6. RT-PCR analysis of transcripts of *hgpA*. The positions of the three primer pairs within the studied locus are shown in the upper panel. Lane 1 is the negative control lacking template DNA. Lanes 2, 3 and 4 use primer set 1; lanes 5, 6 and 7 use primer set 2; lanes 8, 9 and 10 use primer set 3. Lanes 2, 5 and 8 use *H. influenzae* HI689 chromosomal DNA as template; lanes 3, 6 and 9 use RNA sample as a control template; lanes 4, 7 and 10 use cDNA as template. Lane 11 is 100 bp ladder marker.

independent terminator. This sequence analysis suggested that *hgpA* is transcribed as a monocistronic message. RT-PCR was used to investigate this possibility. Random nonamer primers were annealed to RNA prepared from haem-restricted *H. influenzae* HI689, and reverse transcriptase was used to generate cDNA. The cDNA was used as the template for PCR with three pairs of primers (Table 1, Fig. 6) designed such that primer pairs 1 (HFJ2F4 and HFJ16) and 3 (HFJ26 and HFJ29) would amplify PCR products of 650 bp and 660 bp, respectively, if the *hgpA* transcript was polycistronic. The control primer pair 2 (HFJ25 and HFJCTERMS) was designed to amplify an internal 300 bp fragment of *hgpA*. With cDNA as template, only amplification with primer pair 2 resulted in a product, indicating that the *hgpA* transcript is monocistronic (Fig. 6). All primer pairs amplified a DNA fragment of the correct size when genomic DNA was used as template.

Construction of a mutation in *hgpA*

A mutation in *hgpA* was constructed by deletion of the internal 2.5 kbp *Bgl*II fragment and insertion of the 2.2 kbp TSTE antibiotic resistance marker (Sharetsky *et al.*, 1991). That the mutant HI689 strain was correctly constructed was confirmed by Southern analysis (Ren *et*

Fig. 7. Plate bioassay showing utilization of the haemoglobin-haptoglobin complex (4 nmol haemoglobin equivalent) (well 1), haem (0.04 μ mol) (well 2) or haemoglobin (4 nmol) (well 3) by *H. influenzae* HI689 (a) or the insertion/deletion mutant HI689hgp Δ Bg/II (b).

al., 1998). The mutant strain was designated HI689hgp Δ Bg/II.

Haem utilization and binding

The mutant strain HI689hgp Δ Bg/II was unaltered in its ability to grow with either haemoglobin or the haemoglobin-haptoglobin complex as the sole source of haem in plate bioassays (Fig. 7). Following growth in haem-starved conditions the mutant was unaltered in its ability to bind either biotinylated haemoglobin or haemoglobin-haptoglobin complex in a dot-blot assay compared to the wild-type strain HI689 (data not shown).

Affinity isolation of haemoglobin-binding proteins from an *hgpA* mutant

Since there was no apparent alteration in either utilization or binding of haemoglobin or haemoglobin-haptoglobin, bacteria grown in haem-restricted media to mid-exponential phase were subjected to affinity purification using biotinylated haemoglobin as the primary ligand. The wild-type strain HI689 resulted in isolation of an approximately 120 kDa band, whilst the

Fig. 8. SDS-PAGE (7.5%) gel stained with Coomassie blue. Lanes: 1, affinity-purified outer-membrane protein from HI689hgp Δ Bg/II; 2, affinity-purified outer-membrane protein from HI689; 3, molecular mass markers. Arrow A points to a band at approximately 120 kDa in lane 2; arrow B points to a band at approximately 115 kDa present in lane 1 and in lower amounts in lane 2. Numbers on the right indicate molecular masses in kDa.

mutant strain HI689hgp Δ Bg/II did not yield the 120 kDa band; however, a band at approximately 115 kDa was present in increased amounts (Fig. 8). Although the 115 kDa band has been detected at low levels in the wild-type strain, the band is clearly below the limit of Coomassie detection in some affinity isolations performed with the wild-type strain (Jin *et al.*, 1996).

DISCUSSION

H. influenzae requires a porphyrin source for growth (Evans *et al.*, 1974). However the availability of this essential nutrient is strictly limited within the human host. *H. influenzae* does not produce siderophores, and the mechanism(s) by which *H. influenzae* acquires haem and/or iron has not been fully elucidated. Proteins binding the haem-haemopexin complex, of which one is apparently secreted into the growth media, have been described (Cope *et al.*, 1994, 1995, 1998; Hanson *et al.*, 1992; Wong *et al.*, 1995). Haem-binding outer-membrane proteins have been isolated (Lee, 1992; Thomas *et al.*, 1998), and a haem-binding lipoprotein with significant homology to a periplasmic transport protein of *E. coli* has also been characterized (Hanson & Hansen, 1991). In addition, Reidl & Mekalanos (1996) have recently shown that protein *e*(P4) of *H. influenzae* is essential for utilization of haem, protoporphyrin IX and haem from haemoglobin. A short haem-binding motif homologous to regions of other haem-binding proteins was identified in protein *e*(P4) (Reidl & Mekalanos, 1996).

The acquisition and utilization of haem from haemoglobin, the haemoglobin-haptoglobin complex and the haem-haemopexin complex is dependent on a

functional *tonB* gene, indicating that uptake is mediated by an outer-membrane TonB-dependent protein(s) (Postle, 1990; Jarosik *et al.*, 1994). In this study the complete nucleotide sequence of the gene encoding a haem-repressible 120 kDa haemoglobin and haemoglobin-haptoglobin-binding protein (HgpA) of *H. influenzae* type b was determined. HgpA exhibits significant homology with other iron- and haem-related bacterial TonB-dependent proteins over regions that are highly conserved among this class of proteins (Lundrigan & Kadner, 1986). TonB-dependent outer-membrane protein receptors have been reported in a number of bacterial species, and are commonly involved in transport of iron and haem across the bacterial membrane (Bitter *et al.*, 1991; Cornelissen *et al.*, 1992; Gray-Owen & Schryvers, 1995; Lundrigan & Kadner, 1986; Pressler *et al.*, 1988; Maciver *et al.*, 1996). TonB is believed to act in energy transduction from the cytoplasmic membrane to the outer membrane (Postle, 1990; Larsen *et al.*, 1994). HgpA did not contain a region homologous to the haem-binding motif identified in protein *e*(P4) by Reidl & Mekalanos (1996).

Whilst RdKW20 chromosome has homology to the DNA sequences flanking *hgpA*, there is no homologous *hgpA* at this locus, which instead contains the ORFs of HI0588, HI0589, HI0590, HI0591 and HI0592 (Fleischmann *et al.*, 1995). Southern hybridization analyses indicate that a 6.6 kbp DNA sequence present at this position in Rd KW20 is missing in both HI689 and TN106 (Fig. 5).

There are four ORFs in the *H. influenzae* strain Rd KW20 chromosomal sequence, HI0635, HI0661, HI0712 and HI1566, with significant homology to *hgpA* (Fleischmann *et al.*, 1995; Hood *et al.*, 1996). All four ORFs in Rd KW20 and *hgpA* contain multiple CCAA repeats, where the number of repeats in each published reported gene varies between 19 and 37 (Table 2). Our data indicate that the number of CCAA repeats in a given locus may vary in *H. influenzae* (Fig. 3). We propose that the CCAA repeat region may regulate expression of these genes through a slip-strand mechanism. Such a mechanism may modulate expression of proteins in different host sites depending on the prevalent haem source. Alternatively, strand slippage may provide a mechanism to avoid the immunological response of the host. A similar mechanism has been demonstrated to mediate phase variation of the *H. influenzae* *lic* locus (Szabo *et al.*, 1992). High *et al.* (1996) have recently shown that the CAAT repeat motif in the *lic* locus is required for phase variation but not for biosynthesis of lipo-oligosaccharide. The PCR amplification across a CCAA region from different colonies of the same strain yielded varying sized products (Fig. 3). These data provide evidence for the proposed slip-strand mechanism in *H. influenzae*.

Insertional inactivation of *hgpA* did not abrogate the ability of the mutant strain to either bind haemoglobin or the haemoglobin-haptoglobin complex, or to utilize either as a sole haem source. Affinity purification of

haemoglobin-binding proteins from the mutant strain demonstrated the loss of the 120 kDa protein and increased isolation of a 115 kDa protein. These data suggest that at least one more haemoglobin-binding protein exists. We have shown that *H. influenzae* HI689 contains a HI0661 homologue, *hgpB*, which encodes a 115 kDa haemoglobin and haemoglobin-haptoglobin-binding protein (Ren *et al.*, 1998). Elkins *et al.* (1995) also identified a 115 kDa haemoglobin-binding protein from *H. influenzae* strain DL42, although the relationship of this protein to either HgpA, HgpB or the ORFs in strain Rd KW20 is unclear. In some affinity isolations from the wild-type strain HI689, there was no apparent expression of a 115 kDa protein (Jin *et al.*, 1996), consistent with the hypothesis of phase variation of haemoglobin/haemoglobin-haptoglobin-binding protein expression. The mechanism of this apparent shift is as yet unknown, although loss of one protein may result in selection of populations in which strand slippage across the CCAA region has led to expression of an alternative protein.

Haemoglobin-haptoglobin binding was expressed by a fusion of *bhuA* lacking the CCAA repeats, indicating that the repeats are not essential for haemoglobin-haptoglobin binding (Maciver *et al.*, 1996). The clone of *hgpA* bound both haemoglobin (Jin *et al.*, 1996) and the haemoglobin-haptoglobin complex, whereas the clone of *bhuA* bound only the haemoglobin-haptoglobin complex (Maciver *et al.*, 1996). Maciver *et al.* (1996) reported a start site immediately preceding the CCAA repeat units, which would lead to expression of a protein lacking a leader peptide. Removal of a CCAA repeat from their reported nucleotide sequence would bring the protein into frame with an alternative start codon, giving rise to a protein with a leader sequence homologous to that of HgpA.

Mutation of *bhuA* resulted in a decreased ability of the mutant to bind haemoglobin-haptoglobin when grown in a haem-replete medium (Maciver *et al.*, 1996). However, no effect was seen on haemoglobin-haptoglobin binding when bacteria were grown in a haem-restricted medium. The mutant also showed a reduced ability to utilize haemoglobin-haptoglobin as a haem source, but was unaltered in the ability to utilize haemoglobin (Maciver *et al.*, 1996). Thus *bhuA* appears to encode constitutive haemoglobin-haptoglobin binding ability, whilst there is also an independent haem-repressible haemoglobin-haptoglobin-binding activity expressed by this strain. Similarly, there may be constitutive and haem- or iron-repressible haemopexin-binding proteins expressed by *H. influenzae* (Cope *et al.*, 1995; Wong *et al.*, 1994, 1995). In contrast, haemoglobin binding appears to be haem-repressible (Frangipane *et al.*, 1994), although no extensive survey has been reported among organisms grown under haem-replete conditions nor have other conditions been investigated.

The similarity between *hgpA* and *bhuA* is 90%, and *H. influenzae* TN106, the strain from which *bhuA* was cloned, apparently lacks the same 6.6 kbp sequence missing in HI689 (Maciver *et al.*, 1996) (Fig. 5). Thus it

is likely that *hgpA* and *hhuA* represent alleles of the same gene in different strains. However, expression of clones of *hhuA* and *hgpA* in *E. coli* apparently resulted in different phenotypes; *hhuA* bound only the haemoglobin-haptoglobin complex and *hgpA* bound both the complex and haemoglobin alone. Additionally, insertion mutants of *hhuA* and *hgpA* in TN106 and HI689, respectively, result in different phenotypes, with the *hhuA* mutant showing reduced utilization of the haemoglobin-haptoglobin complex, whereas the *hgpA* mutant is unaltered in this respect. Neither mutant is apparently altered in the ability to bind the haemoglobin-haptoglobin complex following haem-restricted growth. These discrepancies may arise from the reported construction of the *hhuA* clone as a fusion protein lacking approximately 150 amino acids at the N-terminal end of the protein (Maciver *et al.*, 1996). It is possible that the missing 150 amino acids are essential for haemoglobin binding, or alternatively that the fusion inhibits haemoglobin binding but not binding of the haemoglobin-haptoglobin complex. These data may reflect differences in the growth techniques, or alternatively may reflect varying complements of haemoglobin and/or haemoglobin-haptoglobin-binding proteins between these strains. In order to elucidate the haemoglobin and haemoglobin-haptoglobin utilization pathways of *H. influenzae*, it will be necessary to define the functions of the additional CCAA-containing ORFs and to define the gene complements of the strains under investigation.

In conclusion, a haemoglobin-binding protein gene, *hgpA*, of *H. influenzae* and several ORFs possessing CCAA repeating units at the 5' ends and encoding putative proteins of high homology to HgpA have been identified. Expression of these genes may be regulated via a slip-strand mechanism. Based on homology at the amino acid level, these proteins are likely to have similar functions. Work is in progress to define the function of each of the gene products, to elucidate the mechanisms of regulation and to define the potential role of the gene products in the pathogenesis of *H. influenzae* disease.

ACKNOWLEDGEMENTS

This work was supported by the Children's Medical Research Institute, Public Health service grant AI29611 from the National Institute of Allergy and Infectious Disease, USA, to T.L.S., and by Health Research contract HN5-055 from the Oklahoma Center for the Advancement of Science and Technology, USA, to D.J.M.

REFERENCES

Bagg, A. & Neilands, J. B. (1987). Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol Rev* 51, 509–518.

van Belkum, A., Scherer, S., van Leeuwen, W., Willemse, D., van Alphen, L. & Verbrugh, H. (1997). Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. *Infect Immun* 65, 5017–5027.

Bezkorovainy, A. (1987). Iron proteins. In *Iron and Infection: Molecular, Physiological and Clinical Aspects*, pp. 27–67. Edited by J. J. Bullen & E. Griffiths. New York: Wiley.

Bitter, W., Marugg, J. D., de Weger, L. A., Tommassen, J. & Weisbeek, P. J. (1991). The ferric-pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. *Mol Microbiol* 5, 647–655.

Cope, L. D., Thomas, S. E., Latimer, J. L., Slaughter, C. A., Muller-Eberhard, U. & Hansen, E. J. (1994). The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. *Mol Microbiol* 13, 863–873.

Cope, L. D., Yogeve, R., Muller-Eberhard, U. & Hansen, E. J. (1995). A gene cluster involved in the utilization of both free haem and haem:haemopexin by *Haemophilus influenzae* type b. *J Bacteriol* 177, 2644–2653.

Cope, L. D., Thomas, S. E., Hrkal, Z. & Hansen, E. J. (1998). Binding of haem-haemopexin complexes by soluble HxuA protein allows utilization of this complexed haem by *Haemophilus influenzae*. *Infect Immun* 66, 4511–4516.

Cornelissen, C. N., Biswas, G. D., Tsai, J., Paruchuri, D. K., Thompson, S. A. & Sparling, P. F. (1992). Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. *J Bacteriol* 174, 5788–5797.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12, 387–395.

Elkins, C., Chen, C. J. & Thomas, C. E. (1995). Characterization of the *hgbA* locus encoding a haemoglobin receptor from *Haemophilus ducreyi*. *Infect Immun* 63, 2194–2200.

Evans, N. M., Smith, D. D. & Wicken, A. J. (1974). Haemin and nicotinamide adenine dinucleotide requirements of *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *J Med Microbiol* 7, 359–365.

Falla, T. J., Dobson, S. R., Crook, D. W. & 7 other authors (1993). Population-based study of non-typable *Haemophilus influenzae* invasive disease in children and neonates. *Lancet* 341, 851–854.

Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496–512.

Frangipane, M. E., Morton, D. J., Wooten, J. A., Pozsgay, J. M. & Stull, T. L. (1994). Binding of human haemoglobin by *Haemophilus influenzae*. *FEMS Microbiol Lett* 118, 243–248.

Gray-Owen, S. D. & Schryvers, A. B. (1995). Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of *Haemophilus influenzae*. *Infect Immun* 63, 3809–3815.

Hanson, M. S. & Hansen, E. J. (1991). Molecular cloning, partial purification, and characterization of a haemin-binding lipoprotein from *Haemophilus influenzae* type b. *Mol Microbiol* 5, 267–278.

Hanson, M. S., Pelzel, S. E., Latimer, J., Muller-Eberhard, U. & Hansen, E. J. (1992). Identification of a genetic locus of *Haemophilus influenzae* type b necessary for the binding and utilization of haem bound to human haemopexin. *Proc Natl Acad Sci USA* 89, 1973–1977.

High, N. J., Jennings, M. P. & Moxon, E. R. (1996). Tandem repeats of the tetramer 5'-CAAT-3' present in *lic2A* are required for phase variation but not lipopolysaccharide biosynthesis in *Haemophilus influenzae*. *Mol Microbiol* 20, 165–174.

Hood, D. W., Deadman, M. E., Jennings, M. P., Bisercic, M., Fleischmann, R. D., Venter, J. C. & Moxon, E. R. (1996). DNA repeats identify novel virulence genes in *Haemophilus influenzae*. *Proc Natl Acad Sci USA* 93, 11121–11125.

- Jarosik, G. P., Sanders, J. D., Cope, L. D., Muller-Eberhard, U. & Hansen, E. J. (1994). A functional *tonB* gene is required for both utilization of haem and virulence expression by *Haemophilus influenzae* type b. *Infect Immun* **62**, 2470–2477.
- Jin, H., Ren, Z., Pozsgay, J. M., Elkins, C., Whitby, P. W., Morton, D. J. & Stull, T. L. (1996). Cloning of a DNA fragment encoding a haem-repressible haemoglobin-binding outer membrane protein from *Haemophilus influenzae*. *Infect Immun* **64**, 3134–3141.
- Klein, J. O. (1997). Role of nontypeable *Haemophilus influenzae* in pediatric respiratory tract infections. *Pediatr Infect Dis J* **16**, S5–S8.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Larsen, R. A., Wood, G. E. & Postle, K. (1994). The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein. *Mol Microbiol* **12**, 857.
- Lee, B. C. (1992). Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type b. *Infect Immun* **60**, 810–816.
- Lee, B. C. (1995). Quelling the red menace: haem capture by bacteria. *Mol Microbiol* **18**, 383–390.
- Lundrigan, M. D. & Kadner, R. J. (1986). Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*. Homology among outer membrane receptors that interact with TonB. *J Biol Chem* **261**, 10797–10801.
- Maciver, I., Latimer, J. L., Liem, H. H., Muller-Eberhard, U., Hrkal, Z. & Hansen, E. J. (1996). Identification of an outer membrane protein involved in utilization of haemoglobin-haptoglobin complexes by nontypeable *Haemophilus influenzae*. *Infect Immun* **64**, 3703–3712.
- Madorne, D. V., Johnson, C. L., Phipps, D. C., Pennridge Pediatric Associates, Popejoy, L. A., Eby, R. & Smith, D. H. (1990). Safety and immunologic response to *Haemophilus influenzae* type b oligosaccharide–CRM197 conjugate vaccine in 1 to 6-month-old infants. *Pediatrics* **85**, 331–337.
- Murphy, T. V., White, K. E. & Pastor, P. (1993). Declining incidence of *Haemophilus influenzae* type b disease since introduction of vaccination. *JAMA (J Am Med Assoc)* **269**, 246–248.
- Otto, B. R., Verweij-van Vught, A. M. & MacLaren, D. M. (1992). Transferrins and haem-compounds as iron sources for pathogenic bacteria. *Crit Rev Microbiol* **18**, 217–233.
- Postle, K. (1990). TonB and the gram-negative dilemma. *Mol Microbiol* **4**, 2019–2025.
- Pressler, U., Staudenmaier, H., Zimmermann, L. & Braun, V. (1988). Genetics of the iron dicitrate transport system of *Escherichia coli*. *J Bacteriol* **170**, 2716–2724.
- Reidl, J. & Mekalanos, J. J. (1996). Lipoprotein *e*(P4) is essential for hemin uptake by *Haemophilus influenzae*. *J Exp Med* **183**, 621–629.
- Ren, Z., Jin, H., Morton, D. J. & Stull, T. L. (1998). *hgpB*, a gene encoding a second *Haemophilus influenzae* hemoglobin- and hemoglobin-haptoglobin-binding protein. *Infect Immun* **66**, 4733–4741.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sharetsky, C., Edlind, T. D., LiPuma, J. J. & Stull, T. L. (1991). A novel approach to insertional mutagenesis of *Haemophilus influenzae*. *J Bacteriol* **173**, 1561–1564.
- Spenser, H. T. & Herriott, R. M. (1965). Development of competence in *Haemophilus influenzae*. *J Bacteriol* **90**, 911–920.
- Stull, T. L. (1987). Protein sources of haem for *Haemophilus influenzae*. *Infect Immun* **55**, 148–153.
- Szabo, M., Maskell, D., Butler, P., Love, J. & Moxon, R. (1992). Use of chromosomal gene fusions to investigate the role of repetitive DNA in regulation of genes involved in lipopolysaccharide biosynthesis in *Haemophilus influenzae*. *J Bacteriol* **174**, 7245–7252.
- Thomas, C. E., Olsen, B. & Elkins, C. (1998). Cloning and characterization of *tdhA*, a locus encoding a TonB-dependent haem receptor from *Haemophilus ducreyi*. *Infect Immun* **66**, 4254–4262.
- Turk, D. C. (1984). The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* **18**, 1–16.
- Wallace, R. J., Musher, D. M. & Martin, R. J. (1978). *Haemophilus influenzae* pneumonia in adults. *Am J Med* **64**, 87–93.
- Weiser, J. N., Love, J. M. & Moxon, E. R. (1989). The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. *Cell* **59**, 657–665.
- Whitby, P. W., Sim, K. E., Morton, D. J., Patel, J. A. & Stull, T. L. (1997). Transcription of genes encoding iron and haem acquisition proteins of *Haemophilus influenzae* during acute otitis media. *Infect Immun* **65**, 4696–4700.
- Wong, J. C., Holland, J., Parsons, T., Smith, A. & Williams, P. (1994). Identification and characterization of an iron-regulated haemopexin receptor in *Haemophilus influenzae* type b. *Infect Immun* **62**, 48–59.
- Wong, J. C. Y., Patel, R., Kendall, D., Whitby, P. W., Smith, A., Holland, J. & Williams, P. (1995). Affinity, conservation, and surface exposure of haemopexin-binding proteins in *Haemophilus influenzae*. *Infect Immun* **63**, 2327–2333.

Received 7 September 1998; revised 8 December 1998; accepted 14 December 1998.